

Antagonistic interactions between *Aureobasidium pullulans* and *Fusarium culmorum*, a fungal pathogen of winter wheat

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Abstract The effect of the biological control agent *Aureobasidium pullulans* (de Bary) G. Arnaud on the development of *Fusarium* head blight (FHB) on winter wheat and kernel contamination with fungi of the genera *Fusarium*, *Acremonium*, *Cladosporium* and *Penicillium* was analyzed in a greenhouse experiment. Scanning electron microscopy was used to evaluate the distribution of *A. pullulans* cells and aggregates on wheat kernels, infection structures of *Fusarium culmorum* (W.G. Smith) Sacc and the antagonist-pathogen interactions. Biological control with *A. pullulans* reduced FHB severity by 21.67 % and improved grain filling by 5.02 %, compared with the control treatment. The survival of *A. pullulans* was good (to 31 cells per kernel), in particular on the surface and in the crease of kernels, including in pathogen-inoculated wheat plants. *A. pullulans* cells firmly adhered to *F. culmorum* hyphae, and damaged them. In most cases, autochthonous communities of filamentous fungi of

the genera *Acremonium* and *Penicillium* developed at a slower rate after kernel inoculation with the pathogen.

Keywords *Aureobasidium pullulans* · *Fusarium culmorum* · Infection structures · Wheat grain · Scanning electron microscopy

Introduction

Aureobasidium pullulans (de Bary) G. Arnaud is a saprotrophic fungal species commonly found in various environments, such as the leaves of crop plants (Grube et al. 2011), cereal grains (Wachowska et al. 2013), fruits (Tournas and Uppal Memon 2009), living quarters (Prasongsuk et al. 2005), food products (Berghofer et al. 2003) and animal feeds (Olstrop et al. 2010). The species is characterized by high morphological plasticity, and Zalar et al. (2008) divided *A. pullulans* strains into six morphologically and genetically different groups. *A. pullulans* is known for its biotechnological significance as a producer of the biodegradable extracellular polysaccharide (EPS) pullulan (poly- α -1,6-maltotriose), which is used in food production (Kuan-Chen et al. 2011; Donot et al. 2012). The biotechnological potential of *A. pullulans* is also associated with its high enzymatic activity. Depending on temperature, type of fermentation, carbon source, pH and water availability, the isolates

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of *A. pullulans* produce endoglucanase, xylanase, beta-glucosidase (Leite et al. 2008), polygalacturonases (Stratilová et al. 2006), amylase (Li et al. 2007; Manitchotpisit et al. 2011) and chitinases (endochitinase, chitobiosidase) and beta-1,3-glucanase that is produced in culture and apple wounds (Castoria et al. 2001). The enzymes produced by *A. pullulans* are involved in mode of action of this microorganism and play a key role in the biological control of plant diseases, suppressing the growth of phytopathogens. *A. pullulans* has been found effective against *Botrytis cinerea* Pers, *Penicillium expansum* Link, *Rhizopus stolonifer* (Ehrenb.) Vuill and *Aspergillus niger* Tiegh on table grapes (Castoria et al. 2001), and the ochratoxigenic *Aspergillus carbonarius* (Bainier) Thom. on table grapes and strawberry (Lima et al. 1997; Castoria et al. 2001; De Felice et al. 2008; De Curtis et al. 2012b), against *B. cinerea* and *P. expansum* on apples (Castoria et al. 2001; Ippolito et al. 2000; Mounir et al. 2007), against three species of the genus *Monilinia* on stone fruit (Mari et al. 2012). De Felice et al. (2008) observed for the first time that three strains of *A. pullulans* were the effective biocontrol agents in reducing both severity of *Aspergillus* rots in the vineyard and ochratoxin A (OTA) accumulation in wine grapes. In this regard these strains were shown to degrade OTA to OTA (de Felice et al. 2008; De Curtis et al. 2012b). Moreover, the strain LS30 of *A. pullulans* significantly reduced powdery mildew on winter wheat under field conditions (De Curtis et al. 2012a). Lytic enzymes produced by yeasts destroy the cell walls of pathogenic fungi. *Candida guilliermondii* (Castell.) Langeron & Guerr and *Candida oleophila* Montrocher have been found to degrade the cell walls of *B. cinerea* (Saligkarias et al. 2002), whereas the cell-wall degrading enzymes produced by *Pichia guilliermondii* Wick. have proven effective against *Colletotrichum capsici* (Syd. & P. Syd.) E.J. Butler & Bisby (Chanchaichaovivat et al. 2008) and *B. cinerea* (Zhang et al. 2011). No research investigated a parasitic relationship between *A. pullulans* and *Fusarium* pathogens. Antibiosis and hyperparasitism between the species and pathogenic fungi could be used to inhibit the growth and development of the latter on winter wheat kernels, and to reduce mycotoxin contamination of wheat grain.

Fusarium head blight (FHB), one of the most devastating diseases of common wheat (*Triticum aestivum* L.), is especially prevalent in warm and

humid climates. The disease is caused by more than ten different species belonging to the genus *Fusarium*, mostly *Fusarium graminearum* Schwabe and *Fusarium culmorum* (W.G. Sm.) Sacc (Parry et al. 1995). In Eastern Europe, *F. culmorum* is the main causal agent of FHB (Jackowiak et al. 2005; Wiwart et al. 2011). In some years the predominant fungal pathogen on wheat grain is *Fusarium poae* (Peck) Wollenw. but this species exhibits low pathogenicity, and it usually does not reduce wheat yields (Vogelgsang et al. 2008; Wachowska et al. 2013). *Fusarium langsethiae* Torp & Nirenberg, which had not been reported from Eastern Europe in the past, has also recently been isolated from wheat (Łukanowski and Sadowski 2008). In Finland the predominant species was *Fusarium avenaceum* (Fr.) Sacc. (91 %), which was isolated from all samples and some cases the wheat kernels were infected with more than one species (Logrieco et al. 2002). The above is indicative of dynamic changes in the composition and development of *Fusarium* species, related to weather conditions, geographical region, and cultivation practices (Edwards 2004). *F. culmorum* enters the host plant through the penetration of the hyphae into the tissue and secretion of starch-degrading enzymes (Jackowiak et al. 2005). In addition, trichothecene biosynthesis is induced in the infection structures of *F. culmorum*. Trichothecenes are able to bind to the 60S ribosomal subunit of eukaryotes, which leads to the inhibition of protein synthesis and induction of apoptosis (Boenisch and Schäfer 2011). Mycotoxins increase the virulence of *Fusarium* pathogens against cereal plants, and pose a significant health risk for humans and animals. Currently, field application of various chemical fungicides is the primary strategy to control these harmful and mycotoxigenic pathogens on maize and on wheat (De Curtis et al. 2011; Haidukowski et al. 2005). Reducing mycotoxin contamination of winter wheat grain below the maximum permissible levels in an important consideration, especially in organic wheat production where the use of fungicides is limited. Previous research has focused on breeding of wheat cultivars of different species with increased resistance to fungal infections (Wiwart et al. 2011), whereas very few studies have focused on possibility of using yeasts for biological control of *Fusarium* diseases (Zhang et al. 2007). The aim of this study was to determine the effectiveness of *A. pullulans* in reducing the severity of winter wheat

spikes infection caused by *F. culmorum*, and to analyze the interactions between *A. pullulans* and *F. culmorum* on winter wheat grain.

Materials and methods

Plant growth in the greenhouse

In September, in the growing seasons of 2009/2010, 2010/2011 and 2011/2012, winter wheat seeds of cv. Bogatka (obtained from breeding company Danko, Choryń, Poland) were placed in pots with a diameter of 30 cm using the typical brown soil formed from clay and sandy loam (World Reference Base for Soil Resources 1998). The soil's pH was equal to 6.5, the carbon content reached a level of 1.05 % and the nitrogen 0.08 %. In the biological control trials, wheat kernels were covered with a cell suspension of *A. pullulans* Ap1 obtained from winter wheat grain by immersing them for five minutes in a suspension with the density of 1×10^8 cells per ml of sterile water. In November, the plants were transferred in soil, in the field for vernalization. In April, the plants were transferred to the greenhouse. Throughout the growing season, the plants were watered every other day. At the tillering stage (BBCH 29) (Weber and Bleiholder 1990), the stem elongation stage (first node, BBCH 31) and the flag leaf stage (BBCH 39) to each pot were applied two grams of NPK fertilizer [Azofoska, INCO, Poland, 1 (N): 0.5 (P₂O₅): 1.4 (K₂O)]. During the growing season, the biocontrol agent was applied at the stem elongation (first node) stage (BBCH 31), and twice at the beginning and end of heading (BBCH 51, BBCH 59) and for the third time at the full flowering stage (BBCH 65). The greenhouse temperature was maintained at 24–28 °C. During flowering (BBCH 65–69), wheat spikes were inoculated with *F. culmorum*. A pot experiment was performed in four replications each consisting of 20 plants.

Biological protection of wheat plants during the growing season

Wheat spikes were protected against fungal pathogens with an *A. pullulans* Ap 1 isolate (Wachowska et al. 2013). Four-day-old colony forming units (CFU) of *A. pullulans* were washed off from the surface of PDA

medium (Merck) with a sterile water. Wheat spikes were sprayed with the resulting suspension with the density of 2×10^8 cells in 1 ml, 6 ml per pot. The biocontrol agent was applied four times during the growing season, at the first node stage (BBCH 31), at the beginning and end of heading (BBCH 51, BBCH 59) and at the full flowering stage (BBCH 65). The control group comprised plants grown from seeds surface disinfected with a 1 % sodium hypochlorite solution, which were sprayed with water four times, at the stem elongation (first node) stage (BBCH 31), at the beginning and end of heading (BBCH 51, BBCH 59) and at the full flowering stage (BBCH 65).

Inoculation of wheat plants with *F. culmorum*

The *F. culmorum* Fc 38 isolate obtained from winter wheat grain was characterized by high virulence, as previously explored against winter wheat seedlings (Wachowska et al. 2013). The conidia of *F. culmorum* were washed off with an inoculation loop, using sterile water, from 14-day-old colonies grown on PDA medium (Merck) in Petri dishes, at 24 °C. The resulting inoculum suspension, with the density of 2×10^4 conidia ml⁻¹ water, was applied onto winter wheat spikes by using two methods. The first method was employed in the growing seasons of 2009/2010 and 2010/2011. The conidial suspension was sprayed onto all spikes, at 6 ml per pot, at the full flowering stage (BBCH 65). Inoculated spikes showed no visible symptoms of FHB. The second method was used in the growing seasons of 2010/2011 and 2011/2012. The conidial suspension was applied to two spikes per pot, at 0.3 ml per spike, by spraying, at the beginning of flowering (BBCH 61). Inoculated spikes were covered with plastic bags, which were removed seven days after inoculation, when visible signs of infection were observed.

Microbiological analysis of winter wheat kernels by scanning electron microscopy (SEM)

Kernel samples were collected at the fully ripe stage (BBCH 89). After fixation (2.5 % glutaraldehyde, 4 °C) and dehydration, wheat kernels were subjected to critical-point drying (CDP 030, BALTEC), mounted, and coated with gold (Fine Coater, JCF–

1200, JEOL, Japan). The samples were analyzed by SEM (JSM–5310LV, JEOL, Japan), at 10–15 kV.

Evaluation of winter wheat yield and mycological analysis of wheat kernels

The spikes harvested at the fully ripening stage (BBCH 92) were threshed, the kernels were weighed and subjected to mycological analysis. Yeasts and filamentous fungi were isolated from wheat grain without visible disease symptoms, and from grain damaged by *F. culmorum*. In order to wash off epiphytes from wheat kernels, random grain samples of 10 g each were placed in 250 ml flasks containing 90 ml sterile water, and were shaken for 30 min (180 rpm) using a laboratory shaker (358-S, Elpin + , Poland). Microbial suspensions were transferred onto Petri dishes (9 cm diameter, 0.1 ml in volume Margomed, Poland). Martin's medium (Martin 1950) warmed to 42 °C was poured into the dishes. The experiment was performed in four replicates. The number of fungal colonies in plates was counted. The colonies of filamentous fungi were identified to the genus or species level based on their sporulation characteristics (Ellis 1971; Leslie et al. 2006) under a light microscope (Nikon E 200, Japan).

Evaluation of FHB severity

Winter wheat spikes were rated for FHB severity at the hard dough stage (BBCH 87). The infection rates of all spikes were estimated as an average percentage of affected surface area according to Bulletin (1998).

Statistical analysis

The data regarding the abundance of microbial communities were log transformed (CFU + 1). Averaged number of cells and cell aggregates of *A. pullulans* per one kernel was given. The significance of differences between mean values was estimated by two-way ANOVA, and the significance of differences between the average population sizes of yeasts, filamentous fungi, cells and cell aggregates of *A. pullulans* on wheat kernels was estimated by the multiple Student–Newman–Keuls (SNK) test using Statistica 10.0 software (StatSoft 2011).

Results

Abundance of yeasts and filamentous fungi on winter wheat kernels

The method of spike inoculation with *F. culmorum* had a significant effect on the growth of yeast populations (Table 1). The abundance of epiphytic yeasts was by almost 34 % lower on wheat kernels collected from inoculated spikes covered with plastic bags, which showed visible symptoms of FHB, in comparison with biologically protected and non-inoculated kernels. Yeast counts were reduced by 16.13 % on wheat kernels inoculated by the first method and showing no signs of infection. The abundance of epiphytic yeasts on kernels harvested from biologically protected and non-inoculated wheat plants was by 56.7 % higher, compared with the control treatment.

In the control treatment, numerous colonies of filamentous fungi were washed off from the surface of wheat kernels, mostly saprotrophic species belonging to the genera *Acremonium*, *Penicillium* and *Cladosporium* (mostly *Cladosporium herbarum* (Pers.) Link and *Cladosporium cladosporioides* (Fresen.) G.A. de Vries). Fungi belonging to the genus *Penicillium* were not isolated from wheat kernels collected from spikes inoculated with *F. culmorum* by the method 1 (Table 1). On kernels from spikes inoculated by the method 2, the community of the above saprotrophs was reduced by 20.17 %, compared with the control. In comparison with control, the inoculation method 1 contributed to a decrease in the abundance of *Acremonium* and *Cladosporium* species, by 39.4 and 32.86 %, respectively, and to an increase in the population size of *Alternaria alternata* (Fr.) Keissl. and *F. poae*, 3.7-fold and by 37.68 %, respectively. No colonies of *F. culmorum* were isolated from kernels derived from inoculated spikes by the method 1. A high moisture content of spikes after inoculation with the pathogen by the method 2 supported the growth of species from genera *Acremonium* and *Cladosporium*. Few colonies of *F. culmorum* were isolated from wheat kernels from heads inoculated by the method 2. The biocontrol agent increased the abundance of *Acremonium* species and *A. alternata* on wheat kernels, by 43.43 and 274.07 %, respectively, and it reduced the growth of *Penicillium* species by 8.83 %, compared with the untreated kernels.

Table 1 Abundance of fungal colonies isolated from the surface of winter wheat kernels

Inoculation	Yeasts	<i>Acremonium</i> spp.	<i>Alternaria alternata</i>	Log (CFU + 1) (\pm SE) per 1 g of grain				<i>F. culmorum</i>	<i>F. poae</i>	<i>Penicillium</i> spp.
Non-treated control	2.61 (± 0.09) ^e	1.98 (± 0.36) ^{b-d}	0.27 (± 0.18) ^a	2.10 (± 0.07) ^d	0.00 (± 0.00) ^a	0.69 (± 0.69) ^{ab}	2.38 (± 0.06) ^d			
<i>F. culmorum</i> method 1	3.43 (± 0.02) ^g	1.20 (± 0.32) ^{a-d}	1.00 (± 0.39) ^{a-d}	1.41 (± 0.39) ^{a-d}	0.00 (± 0.00) ^a	0.95 (± 0.27) ^{a-d}	0.00 (± 0.00) ^a			
<i>F. culmorum</i> method 2	-2.70 (± 0.10) ^e	2.24 (± 0.15) ^d	0.00 (± 0.00) ^a	2.28 (± 0.03) ^d	0.55 (± 0.19) ^{ab}	0.18 (± 0.19) ^a	1.90 (± 0.24) ^{b-d}			
<i>A. pullulans</i>	4.09 (± 0.09) ^b	2.84 (± 0.01) ^d	0.74 (± 0.13) ^{a-c}	2.12 (± 0.34) ^d	0.37 (± 0.21) ^a	0.75 (± 0.25) ^{a-c}	2.17 (± 0.06) ^d			

Means signed by the same letter differ not significantly according to SNK test at $p \leq 0.01$ ($F_{(18, 84)} = 5.35$, $p < 0.0001$ for interaction of two factors)

Distribution of *A. pullulans* on wheat kernels from spikes inoculated with *F. culmorum*.

The hyphae of *F. culmorum* were sporadically observed on kernels collected from heads inoculated by the method 1 and showed no symptoms of FHB, which supported the rapid development of *A. pullulans*. Individual cells and cell aggregates of *A. pullulans* were unevenly distributed on wheat kernels. In the control, they were found on the surface, in the brush and, less frequently, in the crease of kernels (Table 2). In some cases, the homeostasis was disturbed by the application of *A. pullulans* and *F. culmorum* to wheat spikes. Inoculation with *F. culmorum* by the method 1 did not cause changes in the distribution and abundance of *A. pullulans* on the surface of wheat kernels observed under scanning electron microscope (Table 2). After the application of *F. culmorum* abundance of single-celled *A. pullulans* in the crease of kernels was almost ninefold higher than in the control (Table 2). The application of the pathogen reduced the number of individual cells of *A. pullulans* in the brush of kernels by 25.1 %, in comparison with control. After the application of the yeast *A. pullulans* its cells were found mostly in the crease of kernels, and their abundance in the crease was more than fourfold higher, compared with the control treatment. The biocontrol agent and the pathogen reduced the number of aggregates of *A. pullulans* cells on the surface and in the brush of kernels, in comparison with control. The biocontrol agent contributed to a significant increase in the number of cell aggregates of *A. pullulans* in the crease of kernels. *Aureobasidium pullulans* cells were sporadically observed on wheat kernels after inoculation with *F. culmorum* by the method 2, and in most cases they were attached to the numerous hyphae of the pathogen.

Observations under SEM

Symptoms of *F. culmorum* infection were analyzed only on wheat spikes inoculated by the method 2 by using plastic bags. Numerous hyphae of *F. culmorum* were observed on the lateral sides, in the crease and in the brush of kernels showing symptoms of infection (Fig. 1a). On strongly damaged kernels, numerous hyphae were noted even inside the starchy endosperm

Table 2 Distribution of cells and cell aggregates of *A. pullulans* (\pm SE) on different parts of winter wheat kernels inoculated by the first method (spikes showing no symptoms of FHB)

Treatment	Average number of individual cells			Average number of cell aggregates		
	Kernel surface	Crease	Brush	Kernel surface	Crease	Brush
Non-treated control	31.00 (± 2.00) ^c	1.50 (± 0.96) ^a	21.25 (± 0.05) ^{bc}	1.50 (± 0.59) ^z	0.00 (± 0.00) ^x	1.25 (± 0.63) ^{yz}
Inoculation with <i>F. culmorum</i> method 1	28.00 (± 6.74) ^c	13.33 (± 2.96) ^{abc}	15.83 (± 4.85) ^{abc}	1.33 (± 0.99) ^z	0.33 (± 0.21) ^{xy}	0.00 (± 0.00) ^x
<i>A. pullulans</i>	18.06 (± 1.31) ^{abc}	6.19 (± 1.66) ^{ab}	14.56 (± 3.66) ^c	0.50 (± 0.50) ^{xy}	0.88 (± 0.69) ^y	0.63 (± 0.24) ^{xy}

Means signed by the same letter differ not significantly according to SNK test at $p \leq 0.05$ (a–c for average number of individual cells, x–z for average number of cell aggregates). F-values for average number of individual cells: $F_{(2,27)} = 23.53$, $p < 0.001$ (for a part of kernel factor 1), $F_{(2,27)} = 3.21$, $p = 0.64$ (for a treatment factor 2), $F_{(4,27)} = 3.09$, $p = 0.042$ (for interaction). F-values for average number of cell aggregates: $F_{(2,27)} = 3.66$, $p < 0.046$ (for factor 1), $F_{(2,27)} = 0.69$, $p = 0.511$ (for factor 2), $F_{(4,27)} = 3.23$, $p = 0.036$ (for interaction)

with starch grains whose diameter varied from several to over ten μm , with visible pores characterized by concentric, usually bilayer, structure caused by enzymatic degradation (Fig. 1b, dotted arrows). Microscopic images revealed numerous infection structures (Fig. 2), including infection cushions (ic) with an average diameter of 25 μm (Fig. 2a), lobate appressoria (la), 4.5–6 μm in diameter (Fig. 2a, b, d, and runner hyphae (rh), 1.7 μm in diameter (Fig. 3a, b). Foot structures (fs) with a diameter of 4–6 μm , visible on the electronograms, were often located close to the infection cushions (Fig. 2c). After the removal of the infection structures of *F. culmorum*, penetration pores (pp) with a diameter of 4.5–10 μm , were noted in the outer layer of wheat kernels (Fig. 2d).

Yeast cells were sporadically observed on kernels inoculated with *F. culmorum* by the method 2, and in most cases they were attached to the hyphae of *F. culmorum* (Fig. 3a, dotted arrow). Numerous hyphae covering the surface of kernels included degraded hyphae of *F. culmorum*, with a strongly undulating surface (Fig. 3b dashed arrow).

Fusarium head blight

Wheat spikes sprayed with the inoculum in the first two growing seasons showed no symptoms of FHB. Spikes inoculated by the method 2 were strongly damaged, with 91.25 % spike area affected on average (Fig. 4). The effectiveness of biological control in spikes inoculated by the method 2 was estimated at 21.67 %. Spikes inoculated with *F. culmorum* by the method 1 were characterized by lower filling rates, and

1000 kernel weight was by 46.22 % lower, compared with non-inoculated control (Fig. 4). 1000 kernel weight was four-fold lower in spikes inoculated by the method 2 than in the case of control spikes. Biological control improved grain filling by 5.02 %, in comparison with unprotected plants.

Discussion

The fungal communities isolated from the surface of wheat kernels were typical of the analyzed ecological niche (Laca et al. 2006; Berghofer et al. 2003), and they were dominated by yeasts, followed by filamentous fungi of the genera *Acremonium* and *Penicillium* whose abundance decreased after spike inoculation with *F. culmorum*. In treatments with biological control, yeast communities were dominated by colonies of *A. pullulans* whose characteristics have been described previously (Liu et al. 2009; Gniewosz and Duszkievicz-Reinhard 2008). In our study the high population densities of yeasts on wheat kernels protected them against infection caused by *F. culmorum*. The effectiveness of biological control applied in our study was lower than in a greenhouse test performed by Zhang et al. (2007) who demonstrated that an isolate of *Cryptococcus flavescentis* reduced the severity of spike infection caused by *F. graminearum* by 82.2 %. In a greenhouse experiment carried out by Schisler et al. (2006), the *A. pullulans* AS 55.2 isolate reduced the severity of FHB caused by *Gibberella zeae* (Schwein.) Petch by 84 %. According to the cited authors, the probable mechanism of action of the analyzed isolate involves the biodegradation of

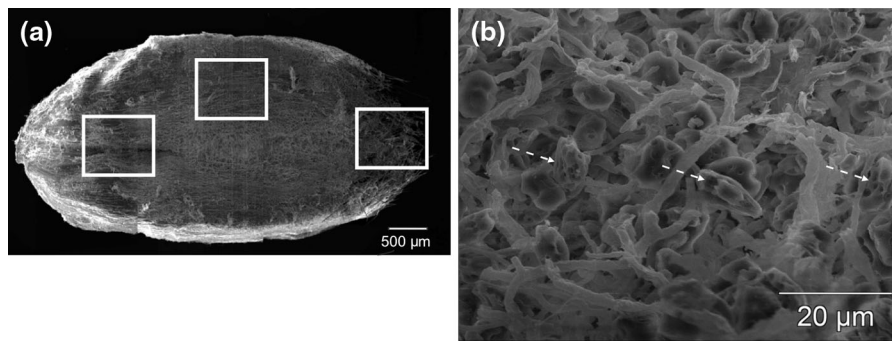


Fig. 1 The areas observed on winter wheat kernels (**a**), damage of starch granules as an effect of the amylolytic enzymes produced by *F. culmorum* (**b**, dashed arrows)

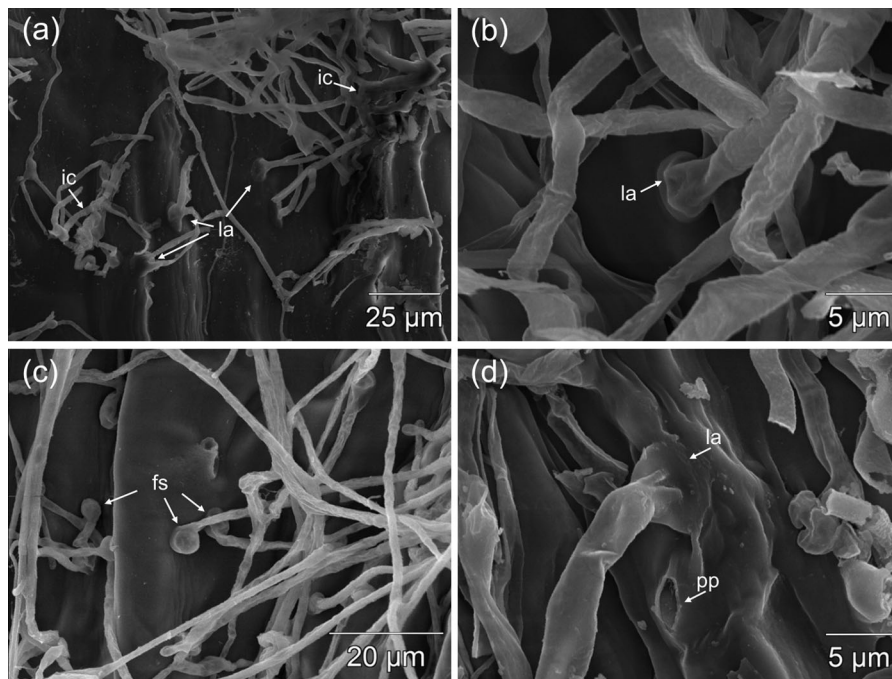


Fig. 2 Infection structures of *F. culmorum* on winter wheat kernels. ic—infection cushion (**a**), la—lobate appressorium (**b**, **d**), fs—foot structures (**c**), pp—penetration pores (**d**)

choline, a compound that stimulates the growth of *G. zeae* (Engle et al. 2004). In a field trial conducted by Khan et al. (2004), the majority of yeast isolates, including members of the genus *Cryptococcus*, suppressed FHB caused by *G. zeae*, but biocontrol efficacy differed depending on wheat cultivar and weather conditions.

In our study the inoculation with *F. culmorum* considerably reduces grain filling rates, by 46.2 and 75 %, by using respectively the first or the second

method in comparison with the control treatment. In a field study by Schade-Schütze et al. (2000), inoculation of wheat spikes with *F. culmorum* reduced 1000 kernel weight by 14–61 %. Korn et al. (2011) demonstrated that the *F. culmorum* isolate was characterized by higher aggressiveness in wheat heads than *F. graminearum* isolates, and that its aggressiveness only partially resulted from the production of mycotoxins such as deoxynivalenol, nivalenol and zearalenone. In our study, higher grain filling rates

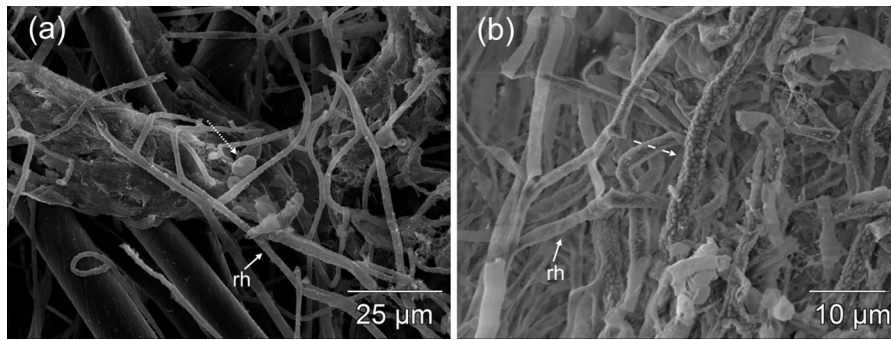


Fig. 3 Yeast cells attached to the hyphae of *F. culmorum* (a, dotted arrow), degraded hyphae of *F. culmorum* (b, dashed arrow). rh—runner hyphae

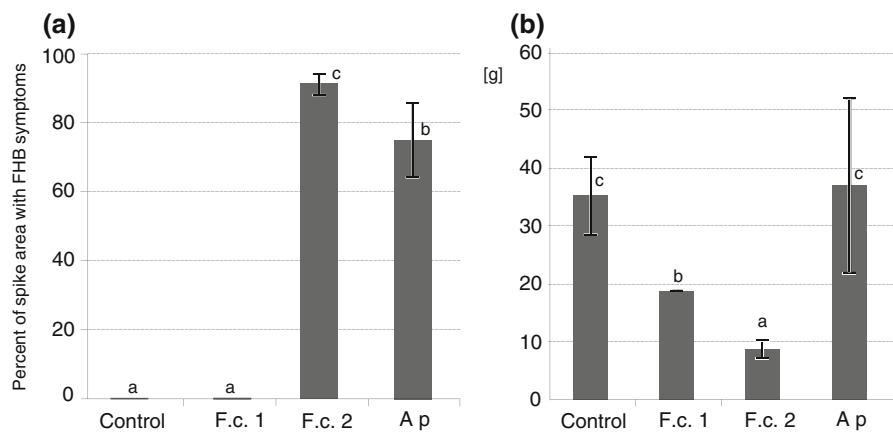


Fig. 4 Severity of FHB symptoms (a) and 1000 kernel weight (b) in conditions of experiment. F.c.1—inoculation with *F. culmorum* method 1, F.c.2—inoculation with *F. culmorum* method 2, Ap—inoculation with *A. pullulans* ($F_{(3,9)} = 36.10$, $p < 0.0001$ for severity of FHB symptoms, $F_{(3,9)} = 17.24$,

$p = 0.00045$ for 1000 kernel weight). Values signed by the same letter differ not significantly according to SNK test at $p < 0.01$ (separately for FHB symptoms and 1000 kernel weight). The error bars correspond to SE

were noted for winter wheat spikes non-inoculated with *F. culmorum* and protected with *A. pullulans*, compared with untreated spikes. Our results corroborate the earlier findings of Khan et al. (2004) who reported that the wheat cultivar Pioneer grown in plots treated with *Cryptococcus* isolates was characterized by higher 1000 kernel weight.

The *A. pullulans* isolate used to protect winter wheat spikes against *F. culmorum* in our greenhouse experiment suppressed this pathogen. Degraded hyphae of *F. culmorum* were observed in few SEM images, and the damage was most likely caused by chitinolytic enzymes. The ability of *A. pullulans* to produce such enzymes has also been reported by other

authors (Govinda Rajulu et al. 2011). In a study by Calistru et al. (1997), SEM images revealed degradation of hyphae of *Aspergillus flavus* Link treated with culture filtrates of *Trichoderma harzianum* Rifai. Minaxi and Saxena (2010) observed that bacteria *Pseudomonas* BAM-4 caused hyphal deformities such as fragmentation, lysis and swelling of mycelia of *Fusarium oxysporum* Schltdl. The changes noted by the cited authors correspond to those observed on the surface of the hyphae of *F. culmorum* in the presented experiment. The adhesion of yeast cells to the pathogen's hyphae, noted in our study, has been described previously (Hashem and Alamri 2009; Zhang et al. 2011; Arras et al. 1999).

We have observed that very few CFU of *F. culmorum* were isolated from severely infected wheat kernels covered with the pathogen's hyphae. This was most likely due to the presence of numerous infection structures that firmly attached the pathogen's hyphae to the surface of kernels, accompanied by very few conidia. The infection structures of *F. culmorum* observed in our study were similar to those described by Boenisch and Schäfer (2011) who investigated the infection process of *F. graminearum*. Numerous SEM images show lobate appressoria and infection cushions of *F. culmorum*—multicellular structures formed by irregularly shaped hyphae. In most cases, the hyphae of *F. culmorum* colonized kernel tissue by immediate penetration (infection hyphae), and less frequently they entered through natural openings. The method I of spike inoculation resulted in the formation of short infection hyphae, described also by Kang and Buchenauer (2000) who analyzed the infection structures of *F. culmorum*. The above authors observed mycelium with infection hyphae on the top of the ovary. Such structures were also frequently encountered in our study. After their removal, SEM images revealed characteristic penetration pores in the outer kernel layer, which suggests that the pathogen produced enzymes capable to degrade the host's tissues. Observed starch granules were also degraded by the enzymes produced by *F. culmorum*. The image of degradation of starch granules observed in our experiment is consistent with that described by Jackowiak et al. (2005)—multilayer pores were noted on the edges and in the center of big (type A) starch grains.

The isolate of *F. culmorum* used in our study exhibited high aggressiveness on winter wheat under greenhouse conditions, but only when inoculated spikes were covered with plastic bags and placed in a moisture chamber. The analyzed isolate formed numerous infection structures, penetrated into the interior of wheat and manifested high enzymatic activity toward the epidermis and starch granules in the kernel. The survival rates of *A. pullulans* on kernels were very high, including in pathogen-inoculated wheat plants, but inoculation shifted the location of its cells and cell aggregates from the brush to the crease of kernels. *A. pullulans* cells firmly adhered to *F. culmorum* cells, and in some cases they damaged the pathogen's hyphae. Autochthonous communities of filamentous fungi of the genera *Acremonium* and

Penicillium developed at a slower rate after inoculation with the pathogen. The growth of *Acremonium* species was suppressed following spike inoculation by the first method, and the growth of *Penicillium* species was inhibited after spike inoculation by the second method.

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